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(57) Abstract

A process for assessing the opsonic function of an individual comprising determining the concentration of mannan binding protein in a body fluid of the individual.

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DIAGNOSTIC PROCEDURE

The present invention is concerned with mannan binding protein (MBP) which is important in the initiation of complement binding and phagocytosis as part of the response to disease organisms. In particular, the invention relates to procedures for detecting the level of MBP in body fluids.

Opsonisation is the process by which foreign agents are coated in order that they may be cleared from the body by phagocytic cells. The failure of serum to opsonise bakers' yeast (Saccharomyces Cerevisiae) for phagocytosis by normal polymorphonuclear leucocytes was first described by Miller et al., Lancet,1968(ii):60-63 in an infant with severe recurrent infections, diarrhoea and failure to thrive. This functional defect was subsequently reported in a series of paediatric patients with frequent unexplained infections (Soothill, Arch. Dis. Child.,51:91-99(1976)), in association with chronic diarrhoea of infancy (Candy et al., Arch. Dis. Child.,55:189-193(1980)) and in association with otitis media in infants (Richardson et al., Arch. Dis. Child.,58:799-802(1983)). A linkage with allergic illness has also been reported (Richardson, loc.cit. and Turner et al., Clin. Exp. Immunol.,34:253-259(1978)). The defect is surprisingly frequent (5-7 %) in the general population, (Soothill,loc.cit., Levinsky et al., J. Immunol. Methods., 24:251-256(1978)) and Kerr et al., Clin. Exp. Immunol., 54:793-800(1983)) and may, therefore, be a factor reducing

the immune potential of such individuals throughout life. Although the defect has been noted, no explanation of the underlying molecular mechanism has been put forward in the literature.

The present inventors have identified a deficiency in serum levels of MBP as being closely correlated with the opsonic defect and have identified the function of MBP in initiating complement binding to foreign agents having mannan-rich or N-acetylglucosamine-rich surfaces. The widespread occurrence of these sugars in the cell walls of pathogenic Gram negative bacteria, mycobacteria and certain yeasts and viruses suggests that low levels of MBP may be linked to a susceptibility to a wide range of common bacterial infection, such as infections by Staphylococcus aureus and Escherichia coli, yeast infections such as Candida albicans and viral infections such as infection with human immuno-deficiency viruses (HIV's), which become clinically manifest through a failure to activate the amplification loop of the complement system.

MBP is a macromolecular calcium dependent animal lectin which has been isolated from the serum and liver of many mammalian species including man, rat, cow and rabbit. The human serum protein has a molecular mass of approximately 700,000 daltons based on Sepharose 6B gel filtration and appears to consist of some 18 subunits each of approximately 32,000 daltons as deduced from other physicochemical

measurements and electron-microscopy. Structurally the protein resembles Clq and comprises three distinct domains: a cysteine-rich amino terminal domain, a collagen-like domain and the carbohydrate recognition domain (CRD) at the carboxyterminus.

In man MBP has been reported to behave as an acute phase reactant (Ezekowitz et al., J.Exp.Med.,167:1034-1046(1988)) and has recently been shown to inhibit in vitro infection of lymphoblasts by the human immunodeficiency virus (HIV) (Ezekowitz et al., J.Exp.Med.,169:185-196(1989). Kuhlman et al., (J.Exp.Med.,169;1733-1745(1989)) have reported that MBP may itself be opsonic, presumably through interactions with a specific Clq-like receptor on the phagocyte surface. However it has not been established that this occurs on a significant scale in vivo since the levels of MBP used in these in vitro assays exceeded the normal level of the protein in serum by two orders of magnitude (i.e. 100-fold).

Based upon their elucidation of the critical role of MBP in opsonisation, the present inventors have developed diagnostic test procedures which allow a patient's opsonic function to be assessed.

Accordingly, in one aspect, the present invention provides a method for assessing the opsonic function of an individual comprising determining the concentration of MBP in a body fluid of the individual.

The body fluid may be any convenient fluid such as blood

or a component thereof, for instance plasma, and is preferably serum. Determination of the MBP concentration in the body fluid may be achieved by any conventional method for quantitating specific proteins, preferably by the techniques which will be described below. Typical serum concentrations of MBP in normal individuals are in the region of 150$\mu g\ l^{-1}$ whereas individuals having 30$\mu g\ l^{-1}$ or less particularly 20 $\mu g\ l^{-1}$ or less are likely to have impaired opsonic function.

In a particular aspect the invention provides a diagnostic process of measuring an individual's MBP levels which process comprises contacting a sample of a body fluid of the individual with an MBP-capture system comprising a solid support and an MBP-capture agent so as to bind MBP to the solid support via the MBP-capture agent and contacting the bound MBP with an MBP-detection system.

Since MBP specifically binds to mannose and N-acetyl glucosamine, any material having a surface rich in either or both of these sugars may be used as the MBP-capture agent. Such materials include mannans (for instance yeast zymosan as described below). Other MBP-capture agents are the sugars N-acetyl-mannosamine, fucose and glucose. The binding of MBP to saccharides such as the foregoing is a calcium-dependant interaction and when any of these saccharides is used as MBP-capture agent the capture medium must contain calcium ions, preferably at a concentration of at least 5 mM. Further MBP-capture agents, which are calcium independent, are polyclonal

or monoclonal anti-MBP antibodies.

The solid support may be any conventional solid support such as multi-well titration plates and polystyrene or latex beads. The solid support may be coated with MBP-capture agent by any conventional method. Preferably the MBP-capture system comprises multi-well titration plates wherein the wells are coated with mannan or an anti-MBP antibody, for instance by the methods described in the Examples below.

The diagnostic process may be conducted by direct detection of the bound MBP or using a competition method in which the MBP in the sample competes for sites on the MBP-capture system with a predetermined amount of a labelled MBP. In the direct process, the MBP-detection system comprises an MBP-recognition agent and means for detecting the binding of the MBP-recognition agent to MBP from the test sample.

At low serum concentrations (e.g. 5%) MBP binding initiates classical pathway activation and C4 fixation which leads to the generation of C3b and which in turn forms a 1:1:1 complex with properdin and factor B. Since C4, C3 properdin and factor B are all components of serum, when the diagnostic process is conducted using the individual's blood or serum, the MBP-capture system will necessarily become coated with C4b and with C3b, properdin and factor B. The MBP-recognition agent may recognise MBP itself or one or more of C4b, C3b, properdin and/or factor B bound thereto. Antibodies against MBP, C4b, C3b, properdin and factor B are

all readily available and may be used as the MBP-recognition agent.

If, for any reason, the diagnostic process is conducted in a medium lacking Complement components, clearly the MBP-recognition agent should be one which directly recognises MBP itself, for instance polyclonal or monoclonal anti-MBP antibodies mannan and saccharides such as N-acetyl mannosamine, fucose and glucose. MBP-recognition agents which directly bind to MBP can also be used when Complement is present in the reaction medium.

Since both MBP-capture and recognition agents rely on specific binding interactions with MBP and the available binding sites on MBP are limited in number, it is preferable that different agents are used as the MBP-capture and -recognition agents.

The MBP-recognition system further comprises means for detecting the binding (directly or indirectly via C3b, properdin and/or factor B) to MBP of the MBP-recognition agent. This may be achieved by any of the conventional labelling techniques. For instance, the label may be linked to the MBP-recognition agent so that the binding of the MBP-recognition agent to MBP may be detected at once. Alternatively, the label may be linked to a specific binding agent which itself recognises and specifically binds to the

bound MBP-recognition agent: An example of this is where the MBP-recognition agent is a rabbit anti-MBP antibody and the labelled specific binding agent is a labelled anti-rabbit IgG antibody; the label is only attached to the MBP-recognition agent once the labelled specific binding agent has been incubated with the complex of MBP and MBP-recognition agent on the solid support.

The labels which may be used in accordance with the present invention are conventional labels such as a radio-labels, fluorescent labels or enzyme labels or labelling systems comprising a specific binding agent (which is linked to the MBP-recognition agent) and its specific binding partner, the latter being linked to a label such as an enzyme label. An example of such a labelling system comprises biotin as the specific binding agent and avidin or, preferably, streptavidin as the specific binding partner.

The diagnostic process of the invention may alternatively be conducted using a competition method, in which case the MBP-detection system comprises MBP linked to a label which is added to the reaction mixture and competes with the individual's MBP for sites on the MBP-capture system. Any conventional label or labelling system as described above may be used.

Labels may be linked to other components of the MBP detection system by conventional techniques. The presence of the label bound to the solid support may be detected by any

conventional method. Quantitation of the label gives a measure of the MBP concentration in the sample of the individual's body fluid.

Suitably the process is conducted on diluted serum, preferably 5% serum, under physiological conditions of temperature and pH, preferably at from 20 to 37°C in buffer at pH from 6 to 8 more preferably at about pH7. The process may be conducted according to any conventional immuno-assay protocol including appropriate washing steps and incubation steps as necessary.

When calcium ions are needed in order for MBP to bind to the capture system, they may be present in the sample of body fluid but are preferably added or supplemented by inclusion of calcium ions in the dilution buffer, preferably at up to 50 mM calcium ions or even more. Suitably the body fluid is diluted to 1 to 20%, preferably about 5% by volume using a dilution buffer containing a calcium salt such as calcium chloride. When calcium ions are needed in order for the MBP-recognition agent to bind to the captured MBP these may be present from an earlier step in the process or they may be added as part of the detection system.

Particularly preferred diagnostic processes according to the present invention are as follows:

(A) The capture system comprises multi-well titration plates coated with mannan, the body fluid is diluted serum and the MBP-recognition system is a rabbit polyclonal anti-

MBP antibody (as MBP-recognition agent) and horse radish peroxidase-labelled anti-rabbit IgG antibody.

(B) The MBP-capture system comprises a multi-well titration plate coated with a polyclonal anti-MBP antibody, the body fluid is diluted serum and the MBP-recognition system comprises biotin-labelled anti-MBP antibody or biotin-labelled mannan (as MBP-recognition agent) and horse radish peroxidase-labelled streptavidin.

(C) The MBP-capture system is as described in either A or B above, the body fluid is diluted serum and the diagnostic process is conducted as a competitive assay using biotinylated MBP and horse radish peroxidase-labelled streptavidin (as MBP-detection system).

(D) The MBP-capture system comprises polyclonal anti-MBP antibody-coated micro-titre plates, the body fluid is diluted serum and the MBP-recognition system comprises mannan (as MBP-recognition agent) linked to biotin and streptavidin-labelled with alkaline phosphatase.

In a particularly preferred embodiment, the diagnostic process is conducted twice using alternative MBP-recognition systems one of which directly recognises MBP itself and the other recognises C3b, properdin or factor B and thereby, indirectly, MBP.

The diagnostic process may further comprise the step of assessing an individual's opsonic function by comparing the MBP concentrations in the body fluid of the individual with MBP levels of normal individuals and/or comparing the MBP

concentration in samples of body fluid of the individual taken at different times during the course of an infective disease. In healthy individuals without MBP deficit, the concentration of MBP in the body fluid will increase during the acute phase of the infection as do other well known acute phase proteins. In individuals with impaired opsonic function, MBP levels remain approximately constant during the acute phase of the infection.

In a further aspect the invention provides a diagnostic test kit comprising an MBP-capture agent and one or more components of an MBP-detection system such as an MBP-recognition agent or labelled MBP. Preferably the kit comprises an MBP-capture system comprising a solid support and, bound thereto, the MBP-capture agent. More preferably the kit comprises the MBP-recognition system including the MBP-recognition agent and any necessary labels or labelled reagents as well as agents for detecting the labels. The diagnostic kit may also contain buffers, controls and standards for use in conducting the diagnostic test process according to the invention.

Low levels of MBP are most likely to have immuno-pathological significance in paediatric patients developing infectious foci at extravascular sites. Within the vascular pool alternative pathway activation mechanisms would operate normally but in the extravascular regions the concentration of serum proteins is much lower and activation of both

classical and alternative pathways may be compromised. MBP deficiency would be a particular risk factor in early infancy when the antibody repertoire is restricted and the levels of potentially compensating IgG opsonins are low. This may explain not only the association of the defect with otitis media in 1 year-old infants but also the apparent compatibility of the defect with health in older individuals. In adults, significantly low levels of MBP have been found in Irritable Bowel Syndrome and may be responsible for difficulties in dealing with yeasts in the gastro-intestinal tract. Low levels of MBP may also be a risk factor in immunocompromised individuals.

The invention will now be illustrated with reference to the figures of the accompanying drawings in which:

Fig. 1 shows that addition of increasing amounts of MBP enriched material containing approximately 0.2 µg/ml MBP (prepared as described in Example 1, Materials and Methods) corrects the opsonic deficiency of serum LB as indicated by measuring the deposition of C4 (-O-), C3b+C3bi (-X-) and factor B (-+-) on a mannan coated plate.

Fig. 2 shows the results of an antibody capture assay for serum MBP:

(a) Levels of MBP in 102 adult blood donors.

(b) Levels of MBP in the sera of ten paediatric patients previously shown to

manifest the opsonic defect.

Fig. 3 shows the results of binding of various complement proteins to mannan coated ELISA plates in a comparative study of 179 sera obtained from healthy blood donors. The plates were developed with specific antibodies as described in Example 2 to reveal the following:

(a) C3bi (C3g specificity)

(b) factor B

(c) properdin

All results are expressed as binding coefficients.

Fig. 4 shows:

(a) Levels of mannan binding protein (MBP) determined in 179 sera from blood donors using the mannan capture assay.

(b) Levels of MBP determined in 102 sera from blood donors using the antibody capture assay.

(c) Correlation between MBP level determined with the mannan capture assay and C3bi binding coefficients.

Fig. 5 shows MBP serum levels in 103 apparently healthy adults as measured by the ELISA technique of Example 3.

The invention is illustrated by the following Examples which are not intended to limit the scope of the invention in any way:

Example 1

MATERIALS AND METHODS

Human sera

Blood samples were obtained with consent from 178 blood donors. Serum was separated from the cells within two hours of bleeding and sub-aliquots frozen rapidly to -70°C. Aliquots were used once only after thawing. Sera from ten paediatric patients were similarly obtained and stored.

Purification of mannan binding protein (MBP)

Four procedures were used sequentially in order to isolate mannan binding protein from (i) a pool of 50ml serum obtained from 100 healthy donors, (ii) 8ml of serum from an individual showing high levels of C3b binding to yeast (subsequently called HB serum), and (iii) 8ml of serum from an individual with low C3b binding activity (subsequently called LB serum).

(i) Sephacryl S300 gel filtration: 500 mM disodium EDTA, pH 8, was added to the serum to give a final concentration of 10 mM EDTA and the serum was fractionated by gel filtration on a column of Sephacryl S300 (85 x 2.6cm)

equilibrated in PBS (Oxoid UK Ltd) buffer, pH 7.3, containing 10 mM EDTA.

(ii) Mannan-Sepharose affinity chromatography: Fractions eluted from the Sephacryl S300 column in the region corresponding to a molecular weight of 700 kDa were dialysed extensively against 40 mM imidazole/HCl pH 7.8 containing 1.25M NaCl. 1M $CaCl_2$ was added to give a final concentration of 50 mM calcium and the fractions were loaded (at room temperature) onto a 3ml mannan-Sepharose affinity column which had been equilibrated with imidazole buffer containing 50 mM calcium (MBB). The column was then washed with this buffer until the OD_{280} was less than 0.02 absorbance units. The bound fraction was eluted with imidazole buffer containing 5 mM EDTA.

(iii) Anti-IgM Sepharose affinity chromatography: The eluate from the mannan-Sepharose affinity column in imidazole buffer with 5 mM EDTA was passed through a Sepharose coupled anti-IgM affinity column and the breakthrough material, depleted of IgM, was used in subsequent purification steps.

(iv) Mono-Q anion exchange chromatography: Unbound material from the anti-IgM affinity chromatography step was concentrated and dialysed against 20 mM Bis Tris pH 6.5

using an Amicon stirred cell (Amicon UK Ltd). The dialysed sample was loaded on to a Mono-Q anion exchange column linked to the FPLC system (Pharmacia UK Ltd) using a start buffer of 20 mM Bis Tris pH 6.5 and a limit buffer consisting of start buffer containing 1M NaCl. MBP enriched fractions were identified by SDS-PAGE (see below) and stored at 4°C.

SDS-PAGE and Western blotting: Purified protein fractions were vigorously reduced by boiling in the presence of 40 mM DTT and duplicate samples were electrophoresed on a 10% reducing SDS-PAGE slab gel by the method of Laemlli (1). The gel was divided; half was silver stained by the method of Morrissey (2), the other half was electroblotted using the BioRad Transblot and probed with rabbit anti-MBP antibody followed by ^{125}I-labelled anti-rabbit Ig (Amersham UK Ltd).

Preparation of anti-MBP antiserum: An antiserum to human MBP was raised in a rabbit using MBP prepared from 6 litres of outdated human plasma. Briefly, the protein was bound to a mannan-Sepharose column, displaced with EDTA, re-chromatographed on a similar, smaller column eluted with mannose and then successively fractionated by Superose 6-gel filtration and Mono-Q ion exchange chromatography. Contaminating IgM was removed using an anti-human IgM affinity column. IgG was isolated from the antiserum by

Na_2SO_4 precipitation and passage of the redissolved precipitate through a DE52 ion exchange column. The antibodies in this preparation were shown to be specific for MBP by probing on a Western blot of whole serum. Further tests established that there was no cross-reactivity with Clq.

Measurement of C3b opsonisation by C3c elution technique

The tryptic release of C3c fragments from zymosan previously incubated with human serum is known to correlate well with other assays of yeast opsonisation. It was possible to calculate a binding coefficient for each sample by including in every assay a serum known to give high levels of C3b binding to zymosan (HB) and a serum known to give low binding in the same system (LB).

$$\text{Binding coefficient (BC)} = 1 - \frac{HB_{C3\%} - Test_{C3\%}}{HB_{C3\%} - LB_{C3\%}} \times 100\%$$

Measurement of complement components bound to mannan: Sera were diluted to 5% in veronal buffered saline containing 5 mM $MgCl_2$ and 5 mM $CaCl_2$ (VBS^{++}) and added to the wells of mannan coated ELISA plates and incubated for 30 minutes at 37°C. The complement fragments which bound to the solid phase were subsequently detected with antibodies specific for C3bi (rat monoclonal anti-C3g), polyclonal anti-C3c (C3b + C3bi specific), anti-factor B and anti-C4 (Serotec UK Ltd,

Kidlington, Oxford).

A serum known to give high levels of C3b binding to yeast, zymosan and mannan (HB) and a serum known to give low levels of binding to the same substrates (LB) were included in every assay and used to calculate a binding coefficient for each test system.

$$\text{Binding coefficient (BC)} = 1 - \frac{HB_{OD492} - Test_{OD492}}{HB_{OD492} - LB_{OD492}} \times 100\%$$

Correction assay: In this assay, small volumes of MBP protein were titrated into the serum with low C3b binding activity (LB) used at 5%. In each case adjustments were made with VBS^{++} to give a constant volume and constant Ca^{++} and Mg^{++} concentrations. The binding coefficients of complement proteins to mannan coated ELISA plates were then measured as above.

Measurement of mannan binding protein (MBP) in serum and column eluates by mannan capture assay: Dynatech Immulon MicroELISA plates were coated overnight at 4°C with mannan (Sigma UK Ltd) diluted to 0.5mg/ml in carbonate/bicarbonate buffer (1.59g/l Na_2CO_3 plus 2.93g/l $NaHCO_3$ pH 9.6). The coated plates were washed three times with PBS-Tween (PBS-T) (PBS pH 7.3 (Oxoid UK Ltd) with 0.05% Tween 20), once with PBS without Tween, and once with imidazole buffer. Sera were diluted to 5% in imidazole buffer (40 mM imidazole/HCl pH 7.8 with 1.25 M NaCl and 50 mM $CaCl_2$) and 100µl aliquots added in

duplicate to the wells of the mannan coated plate which were then incubated at 37°C for two hours. The plates were washed four times with PBS-T and then rabbit anti-human MBP, diluted to 1/500 in PBS-T, was added to all wells and incubated at 37°C for two hours. The plates were washed four times with PBS-T and then incubated at 37°C with horse radish peroxidase-sheep anti-rabbit IgG conjugate (Serotech UK Ltd) at 1/500 in PBS-T. The plates were further washed four times with PBS-T and then o-phenylene diamine solution consisting of 10μg o-phenylene diamine in 20ml citrate-phosphate buffer pH 5.2 (10.4g citric acid plus 14.4g Na_2HPO_4/litre) containing 10μl 30% H_2O_2 was added to all wells and incubated at room temperature in the dark for 15-30 minutes. The colour reaction was stopped by adding 100μl of 4N H_2SO_4 to each well. The plates were read at 492nm using a Titertek Multiskan Plate Reader. An MBP binding coefficient was then calculated as described above for the binding of complement components. A calibrated gravimetric standard serum was also included in the assay and permitted quantitation of MBP levels in $\mu g\ l^{-1}$.

MBP was also assayed using an antibody capture sandwich ELISA in which the rabbit anti-MBP serum was used as both the capture and detector antibody. When used as the detector the antibody was biotinylated by the method of Guesdon et al (3) and the assay developed with streptavidin peroxidase. A pool of serum from healthy adults, previously calibrated against a

purified preparation of MBP, was s rially diluted to provide a standard curve, and unknowns were determined by reference to this. A calibrated gravimetric standard serum was also included in the assay and permitted quantitation of MBP levels in $\mu g\ l^{-1}$.

Statistical analysis: Correlation coefficients (non-parametric Spearman Rank) were determined using the SAS statistical package (SAS Institute Inc, Cary, NC, USA).

RESULTS

Since mannan binding protein is a Ca^{++} dependent lectin, EDTA displacement from a mannan-Sepharose affinity column was chosen as the major purification step. Pre-fractionation of serum on Sephacryl S300 to give material of approximately 700 kDa reduced the protein load on the affinity column and further purification steps (IgM depletion and Mono-Q FPLC) gave preparations which were analysed by SDS-PAGE under reducing conditions. A major subunit of approximately 32 kDa was present in material derived from a 50ml pool of serum (100 donors) and from 8ml of serum obtained from an individual donor previously shown to have high levels of C3b binding activity (HB). In addition a minor 64 kDa band, presumably due to incomplete reduction, was also clearly present in the pooled MBP preparation. When 8ml of serum

from a donor with low levels of C3b binding activity (LB) were fractionated in exactly the same way, there was no evidence of the expected major band at 32 kDa.

Electroblotting and probing with the anti-MBP antiserum revealed both the major 32 kDa and the minor 64 kDa bands in the material derived from a serum pool and the donor with high C3b binding (HB) but no evidence of these bands in material derived from the individual with low C3b binding (LB).

When the MBP enriched material derived from the serum pool was used in the correction assay, it conferred complement binding activity to the LB serum in a dose dependent fashion. The C4, C3b+C3bi and factor B levels observed after titration of MBP into LB are shown in Figure 1.

The MBP levels of 102 blood donors are plotted in Figure 2 Panel (a) and the levels of the protein in ten paediatric patients with the opsonic defect are shown in Panel (b).

The levels of MBP in all ten of this random group of patients were below 30μg/litre. The C3c elution data defining poor opsonic function, MBP levels and deposition of C3 and C4 fragments by sera from each individual patient are shown in Table I. All of these values were either very low or low within the normal range.

Further evidence linking levels of MBP with the opsonic capacity in the population of blood donors is summarised in

Table 1 Levels of mannan binding protein (MBP) in the serum of ten paediatric patients previously shown to have opsonic deficiency.

Patient	Sex	Diagnosis	Functional opsonic assay* Binding Coefficient (%)	MBP (ug/litre)	C3 fragments bound to mannan Binding Coefficient (%)	C4 fragments bound to mannan Binding Coefficient (%)
CS	F	Respiratory tract infections	0.0	10.4	-2.9	-0.6
TC	M	Upper respiratory tract infections	5.9	12.2	-3.6	-0.9
PC	M	Respiratory tract infections (and IgG2 deficiency)	-5.6	5.2	-3.5	-0.8
JF	M	Upper respiratory tract infections	0.0	20.9	0.5	-0.2
SA	F	Pyrexia of unknown origin	0.0	17.4	1.0	9.8
GB	M	Upper respiratory tract infections and migraine	0.0	12.2	-2.0	-0.6
SG	M	Severe diarrhoea, failure to thrive, parotitis with pyrexia	5.3	28.9	-11.4	-0.9
SW	M	Respiratory tract infections and bronchiectasis	0.0	25.0	13.9	1.7
NO	F	Pyrexia of unknown origin	5.3	15.5	-16.4	-1.3
JM	M	Upper respiratory tract infections and failure to thrive	-5.0	18.7	10.5	0.0

No other immunological abnormality was found in these patients other than IgG2 deficiency in patient PC.

C3c elution assay. Expressed as a binding coefficient (see text). Normal range approx. 68.4-110.5%.

Levels determined by antibody capture assay.

Expressed as a binding coefficient (BC). Note that negative binding coefficients were obtained when ELISA colour development was less than that given by the standard serum LB defining low binding. The normal range of binding coefficients for C3 fragments is approx. 30-115% and that for C4 fragments 15-120%.

Table II Correlations between levels of MBP binding to mannan coated ELISA plates (MBP mannan capture assay), C3c elution from yeast (functional opsonic assay), the antibody capture assay for MBP and mannan capture assays for C3bi and C4 in healthy adult blood donors.

			n	Spearman Rank correlation r_s	P value
MBP (mannan capture)	vs	C3c elution (functional opsonic assay)	76	0.4688	<0.0001
MBP (mannan capture)	vs	MBP (antibody capture)	102	0.930	<0.0001
MBP (mannan capture)	vs	C3bi (mannan capture)	178	0.8744	<0.0001
MBP (mannan capture)	vs	C4 (mannan capture)	178	0.8584	<0.0001

Table II which compares results obtained with the mannan capture assay for MBP with those of the functional opsonic assay by C3c elution, the antibody capture assay for MBP, the mannan capture C3bi assay and the mannan capture C4 assay. These studies showed highly significant correlations.

References for Example 1

1. Laemmli, Nature, 227:680-685(1970).

2. Morrissey, Analytical Biochemistry, 117:3097-310(1981).

3. Guesdon et al., J. Histochem. Cytochem., 27:1131-1139(1979).

Example 2

MATERIALS AND METHODS

Measurement of complement components binding to mannan

The binding of various complement proteins to mannan coated ELISA plates was studied using the following protocol. The wells of Immulon Dynatech micro-ELISA plates were filled with 100 µl volumes of mannan (Sigma, Poole, UK; Code No. M-3640 prepared from S. cerevisiae by the Cetavlon method) at 0.5 mg/ml in carbonate/bicarbonate buffer pH 9.6 (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.2 g NaN_3 made up to 1 litre). After incubation overnight at 4°C, the mannan coated plates were washed three times with phosphate buffered saline pH 7.3 (Oxoid UK Ltd) containing 0.5% (v/v) Tween-20 (PBS-T), once with PBS (without Tween-20) and once with VBS.

The serum samples were diluted in Micronic tubes (Flow Laboratories) to 5% in VBS containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$. Duplicate aliquots (100 µl) were then loaded into the wells of the mannan coated ELISA plates and the plates incubated at 37°C for 30 minutes. The plates were then washed four times with PBS-T and bound ligands detected by incubation at room temperature for 1 hour with the following indicator antibodies diluted in PBS-T:

- horseradish peroxidase labelled polyclonal sheep anti-human C3c, and factor B obtained from Serotec

UK Ltd (Kidlington, Oxford). The anti-C3c reagent was used at a dilution of 1/10000, whereas the anti-factor B antibodies were used at 1/2000.

- mouse anti-properdin antibody biotinylated by the method of Guesdon et al., (3) and used at a dilution of 1/4000.
- rat monoclonal anti-C3g biotinylated and used at a dilution of 1/1000.

Following incubation with the indicator antibodies the plates were washed four times with PBS-T. The plates which had been incubated with the anti-properdin and anti-C3g monoclonal antibodies were then incubated for a further hour with streptavidin-peroxidase (Serotec UK Ltd) at 1/4000 and peroxidase labelled sheep anti-mouse IgG (Sigma, Poole, UK) diluted to 1/500 in PBS-T. Following this incubation step the plates were washed four times with PBS-T. Colour was developed following incubation for 15-30 minutes in the dark at room temperature with 100 µl per well of a solution of 10 µg of o-phenylene diamine in 20 ml phosphate-citrate buffer pH 5.2 (prepared from 10.5 g citric acid, 14.2 g Na_2HPO_4 dissolved in 1 litre) containing 10 µl 30% H_2O_2. The colour reaction was stopped by the addition of 4N H_2SO_4 (100 µl per well) and the optical densities evaluated at 492 nm using a Titertek Multiskan ELISA plate reader and the Titersoft Program (Flow UK Ltd).

A serum known to giv high levels of C3b binding to

zymosan (HB) and serum known to give low binding in the same system (LB) were included in every assay and used to calculate a binding coefficient for each test system.

$$\text{Binding coefficient (BC)} = 1 - \frac{HB_{OD492} - Test_{OD492}}{HB_{OD492} - LB_{OD492}} \times 100\%$$

Measurement of mannan binding protein (MBP)

Sera were diluted to 5% in imidazole buffer (40 mM imidazole/HCl pH 7.8 with 1.25 M NaCl and 50 mM $CaCl_2$) and 100 µl aliquots were added in duplicate to the wells of Immulon micro-ELISA plates pre-coated with mannan as above. The plates were then incubated at 37°C for 2 hours, washed four times with PBS-T and rabbit anti-human MBP diluted to 1/500 in PBS-T was added before a further incubation at 37°C for 2 hours. The plates were washed four times with PBS-T and then incubated at 37°C with horseradish peroxidase-sheep anti-rabbit IgG conjugate (Serotech UK Ltd) at 1/500 in PBS-T. The plates were further washed four times with PBS-T and colour developed as above. An MBP binding coefficient was then calculated as described for the binding of complement components.

MBP was also assayed using an antibody capture sandwich ELISA in which the rabbit anti-MBP was used as both the capture and detector antibody. When used as the detector the antibody was biotinylated by the method of Guesdon et al. (3) and the assay developed with streptavidin peroxidase. An MSP

binding coeffici nt was then calculated as above.

Statistical analyses

Pearson product moment correlations between variables were calculated using simple linear curve fit regression analysis and plotted with the Microsoft Chart software (Redmond, WA, USA).

Non-parametric Spearman Rank correlation coefficients were determined using the SAS statistical package (SAS Institute Inc., Cary, NC, USA).

Mannan binding complement components

179 serum samples were available for the determination of mannan binding coefficients using antibodies specific for C3bi, factor B, and properdin and the results obtained are plotted in Figure 3. The results obtained with the anti-C3c antibody (recognising both C3b and C3bi determinants) were very similar to those obtained with the anti-C3bi antibody and are not included in Figure 3.

In each case there was a broadly similar profile of binding activity with a sub-population of individuals in whom the binding coefficients were close to or below zero.

The levels of C3 fragments binding to zymosan in the C3c elution assay were found to correlate significantly with the C3bi mannan binding coefficients when these values were compared in the subpopulation of 77 individuals and there

were strong correlations between all of the mannan binding coefficients for complement proteins determined using sera from the larger population of 179 individuals.

Levels of mannan binding protein (MBP)

Using the mannan capture ELISA system MBP binding coefficients were measured in 179 serum samples from blood donors and sufficient sample was available from 102 of the donors to make similar measurements with the antibody capture assay. These results are illustrated in Figures 4(a) and 4(b).

When the MBP binding coefficients obtained in the capture assay are compared with the levels of C3bi bound to the plates using the same sera, highly significant correlations are observed (Figure 4(c) and Table (III).

Table III

Correlations between various assays using a population of healthy adult blood donors.

		Pearson Correlation		Spearman Rank correlation*	
	n	r	p value	r_s	p value
C3c elution + vs. C3b binding	77	0.672	<0.0001	0.627	<0.0001
C3c elution vs. C4 binding	77	0.488	<0.0001	0.495	<0.0001
C3c elution vs. properdin binding	77	0.656	<0.0001	0.574	<0.0001
C3bi binding v.s properdin binding	178	0.927	<0.0001	0.878	<0.0001
C3bi binding vs. C4 binding	178	0.844	<0.0001	0.867	<0.0001
C4 binding vs. properdin binding	178	0.743	<0.0001	0.741	<0.0001
MBP (mannan capture)vs. C3c elution	76	0.497	<0.0001	0.469	<0.0001
MBP (Mannan capture)vs. MBP (antibody capture)	102	n.d	n.d	0.930	<0.0001
MBP (mannan capture)vs.C3bi binding	178	0.874	<0.0001	0.874	<0.0001
MBP (mannan capture)v.s C4 binding	178	0.854	<0.0001	0.858	<0.0001
MBP (mannan capture)vs. properdin binding	178	0.780	<0.0001	0.736	<0.0001
MBP (mannan capture)vs. factor B binding	178	0.854	<0.0001	0.836	<0.0001
C3bi binding vs. IgG binding	171	0.014	n.s.	0.091	n.s.
C3bi binding vs. IgG binding	173	0.017	n.s.	-0.012	n.s.
C4 binding vs. IgG binding	171	-0.001	n.s.	0.107	n.s.
C4 binding vs. IgM binding	173	-0.028	n.s.	0.016	n.s.
MBP (mannan capture)vs. IgG binding	171	0.019	n.s.	0.064	n.s.
MBP (mannan capture)vs. IgG binding	173	0.109	n.s.	0.092	n.s.

*Since the date shown in Figures 2 and 4 is not normally distributed, the non-parametric Spearman Rank test of correlation has also been used.

+ C3e elution = functional opsonic assay (see text)

n = number of individuals compared

n.s. = not significant ($p>0.05$)
n.d. = not determined

EXAMPLE 3

ELISA for measuring MBP

The assay system used in the present study included a step involving the Ca^{+2}-dependent binding of mannan-biotin to antibody bound MBP prior to incubation with an avidin-linked-indicator enzyme, and subtraction of any background binding due to Ca^{+2}-independent binding. The use of both immunoreactivity and the Ca^{+2} dependent mannan binding step ensured a high specificity for measurement of MBP down to a level of 1ng/ml.

Purified MBP (20μg) was used to immunise a rabbit and the rabbit was given a booster injection of MBP (20 μg) after one month. Blood was taken from the rabbit two weeks after the booster injection and the IgG fraction was purified by sodium sulphate precipitation and ion-exchange chromatography and then stored at 4°C in the presence of sodium azide (0.1%, w/v).

Microtiter plates (Nunc-Immuno plate, GIBCO) were coated at room temperature with rabbit anti-human MBP IgG (100 μl per well of a 10 μg/ml solution) in buffer (sodium carbonate, 15mM, sodium hydrogen carbonate, 35 mM, pH 9.6) for 16 hours and then washed three times with tris-buffered saline, (10 mM tris, 150 mM sodium chloride, 0.05% sodium azide, 0.05% Tween 20, pH 7.4, TBS-NT). Unbound sites were blocked by addition of bovine serum albumin (BSA, 100 μl 1 mg/ml solution in TBS-

TBS-NT) to the wells for 2-3 hours at room temperature followed by washing as before. The plates were stored at 4°C with tris buffered saline (10mM tris, 150mM sodium chloride, 0.05% sodium azide; (TBS-N).

Serum samples were diluted in TBS-NT containing 10mM ethylene diamine tetraacetic acid disodium salt (TBS-NTE); and the dilutions were incubated in the wells for 2 hours at room temperature.

After washing in TBS-NTE, the plates were incubated with a $^1/_{1000}$ dilution of mannan-biotin in TBS-NT containing calcium 20 mM (TBS-NT Ca^{+2}) or TBS-NTE; thus providing calcium dependent and calcium independent binding values. The wells were washed with TBS-NT Ca^{+2} or TBS-NTE and ExtrAvidin-alkaline phosphate conjugate (Sigma E2636), diluted $^1/_{10^4}$ in TBS-NT Ca^{+2}, was added followed by incubation overnight at 4°C.

The plates were then washed with TBS-NT Ca^{+2} and a phosphatase substrate (p-nitrophenyl phosphate disodium; Sigma Chem. Co.) in buffer (50 mM Tris hydrochloride, 5 mM calcium chloride, 5mM magnesium chloride and 0.05% w/v sodium azide, pH 9.1) added to give a final concentration of substrate of 1 mg/ml. The plates were read after incubation for 1 hour at 37°C on a Multiskan MCC/340 (Titertek Co Ltd).

As outlined above, serum samples were routinely incubated with mannan-biotin in both a calcium containing buffer and an EDTA-containing buffer in order to obtain

values for calcium-dependent and calcium-independent binding in addition to the binding dependent upon immunoreactivity. The EDTA controls usually gave very low titres but were always performed and subtracted from the values obtained using Ca^{+2} containing buffers throughout.

The assay was standardised by using a preparation of purified MBP the concentration of which had been determined by amino acid analysis.

Calibration was effected by use of known amounts of purified MBP alone or added to sera showing zero or extremely low levels of MBP, yielding the expected MBP concentration. Assay of these mixtures indicated that the low levels seen with certain sera were not a result of interference with the Ca^{+2} binding or immunoreactivity steps in the assay procedure.

Assay of 103 apparently healthy, adult, blood-donors and laboratory staff showed a wide range of serum MBP levels ranging from 0-870 μg/litre with 90% of the values lying between 1-160 μg/litre (Fig. 5). Identical values were obtained for plasma and serum samples from the same individual and the assay was found to be highly reproducible and accurate down to 1 ng/ml of MBP.

CLAIMS

1. A process for assessing the opsonic function of an individual comprising determining the concentration of mannan binding protein in a body fluid of the individual.

2. A diagnostic process of measuring an individual's mannan binding protein levels which process comprises contacting a sample of a body fluid of the individual with a mannan binding protein capture system comprising a solid support and a mannan binding protein capture agent so as to bind mannan binding protein to the solid support via the mannan binding protein capture agent and contacting the bound mannan binding protein with a mannan binding protein detection system.

3. A process according to claim 2 wherein the capture agent is selected from the mannans, N-acetyl mannosamine, fucose and glucose and the sample is contacted with the capture system in the presence of calcium ions.

4. A process according to claim 2 wherein the capture agent is selected from polyclonal and monoclonal anti-mannan binding protein antibodies.

5. A process according to any one of claims 2 to 4 wherein the solid support is a multi-well titration plate, polystyrene beads or latex beads.

6. A process according to any one of claims 2 to 5 wherein the detection system comprises a mannan binding protein recognition agent and means for detecting

the binding of the recognition agent to mannan binding protein from the sample.

7. A process according to claim 6 wherein the sample is contacted with the capture system in the presence of serum and the recognition agent is selected from mannan binding protein, polyclonal and monoclonal antibodies against mannan binding protein, complement component C4b or C3b, properdin or factor B.

8. A process according to claim 6 wherein the sample is contacted with the capture system in the absence of serum and the recognition agent is selected from mannans, N-acetyl mannosamine, fucose, and glucose and polyclonal and monoclonal antibodies against mannan binding protein.

9. A process according to any one of claims 2 to 8 wherein the capture agent is different from the recognition agent.

10. A process according to any one of claims 2 to 5 wherein the recognition agent comprises mannan binding protein linked to a label and the sample is contacted with the capture system in the presence of the recognition agent.

11. A diagnostic test kit comprising a mannan binding protein capture agent and one or more components of a mannan binding protein recognition system.

Fig.1.

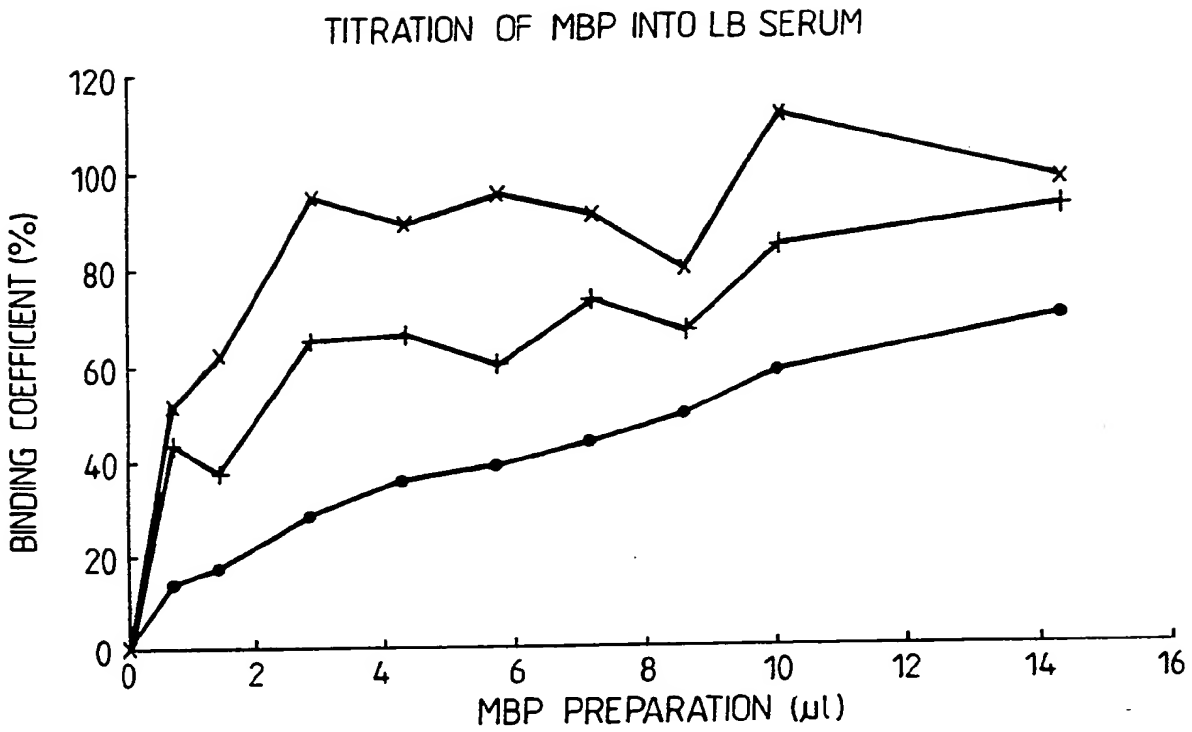


Fig.5.

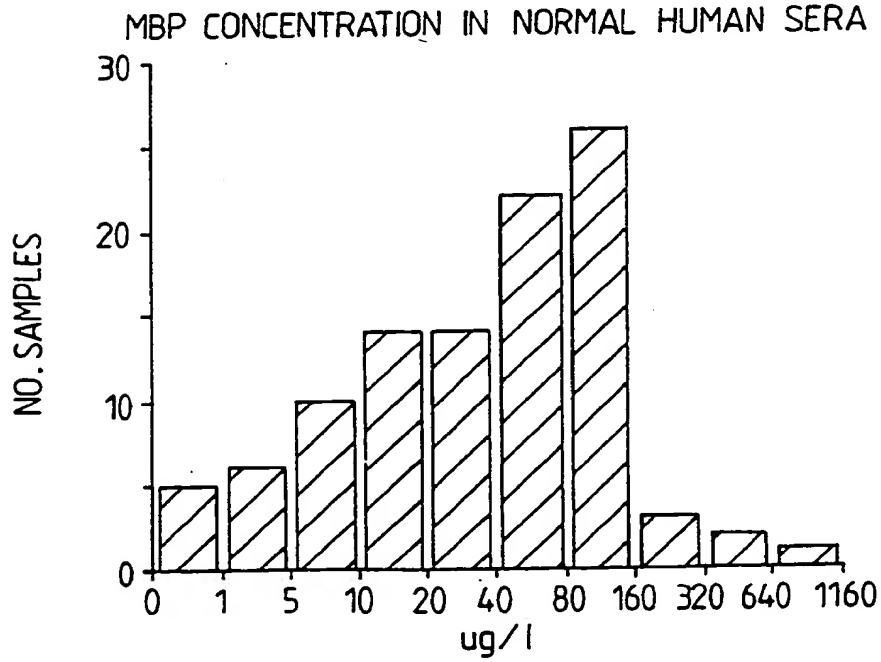


Fig.2.

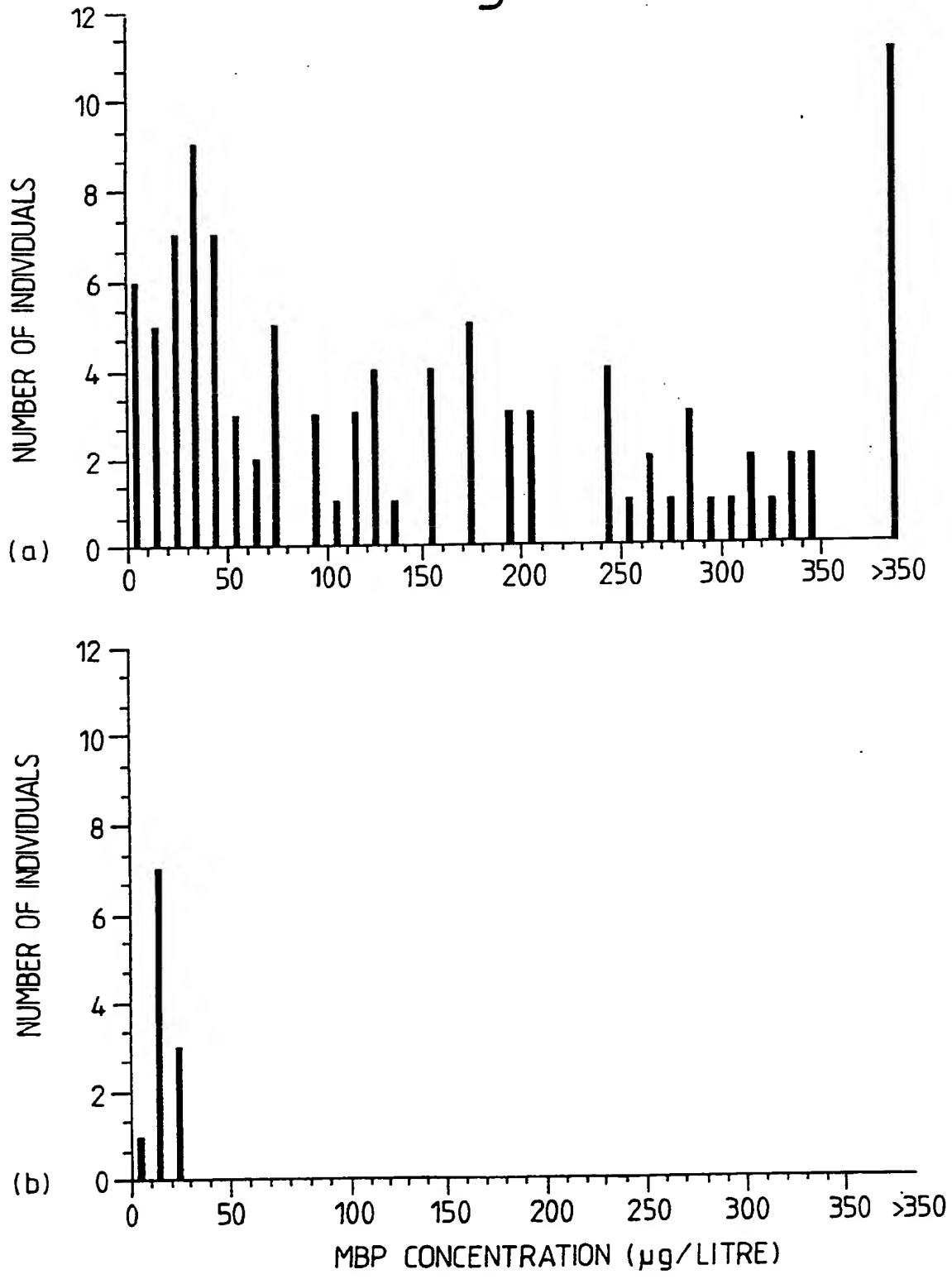
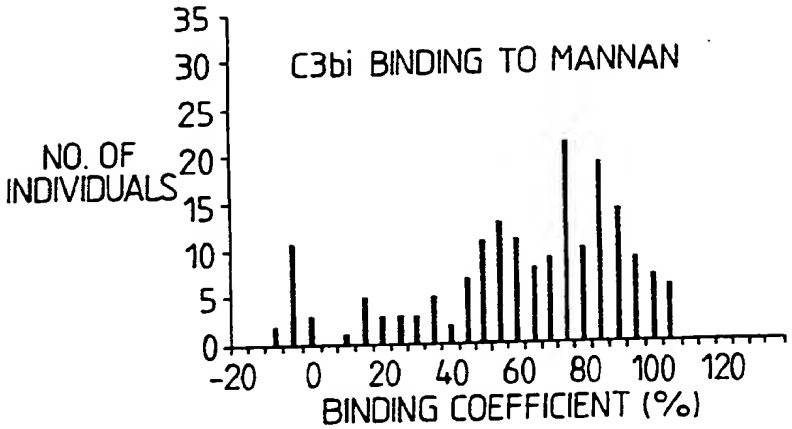
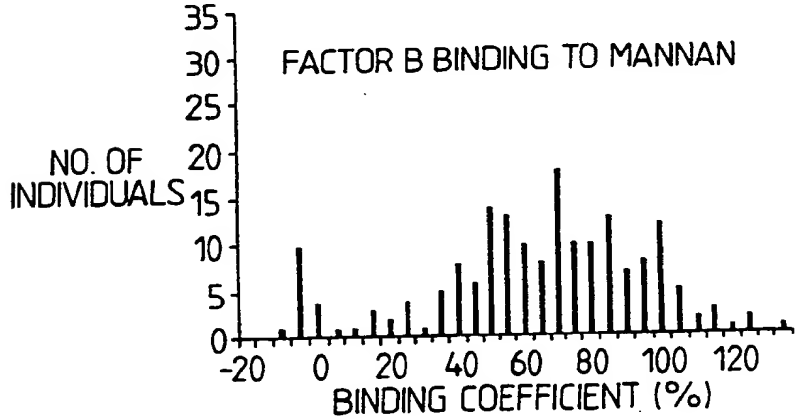


Fig.3.

(a)



(b)



(c)

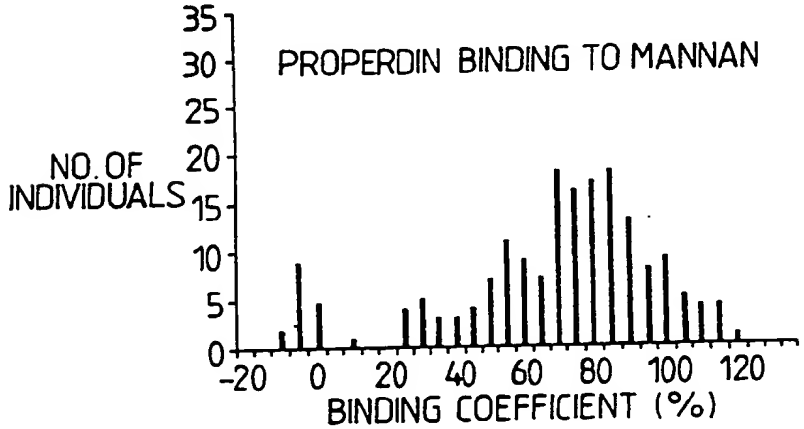


Fig.4.

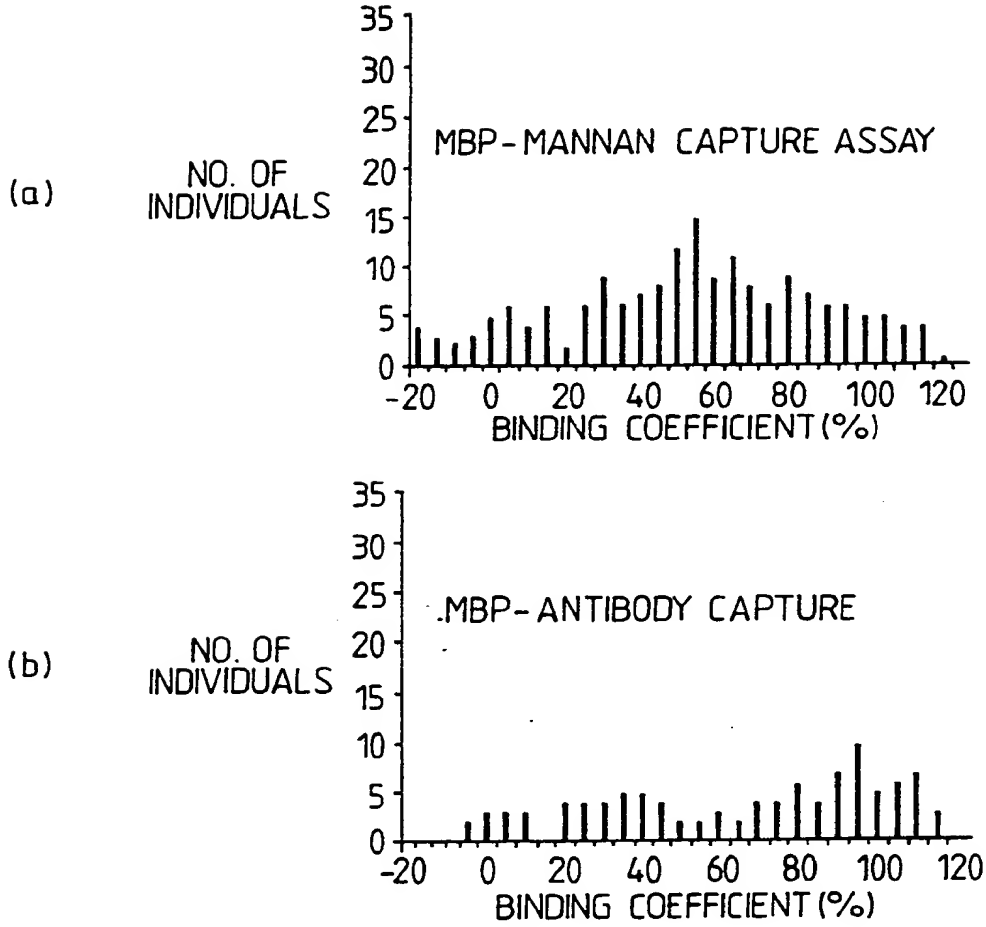
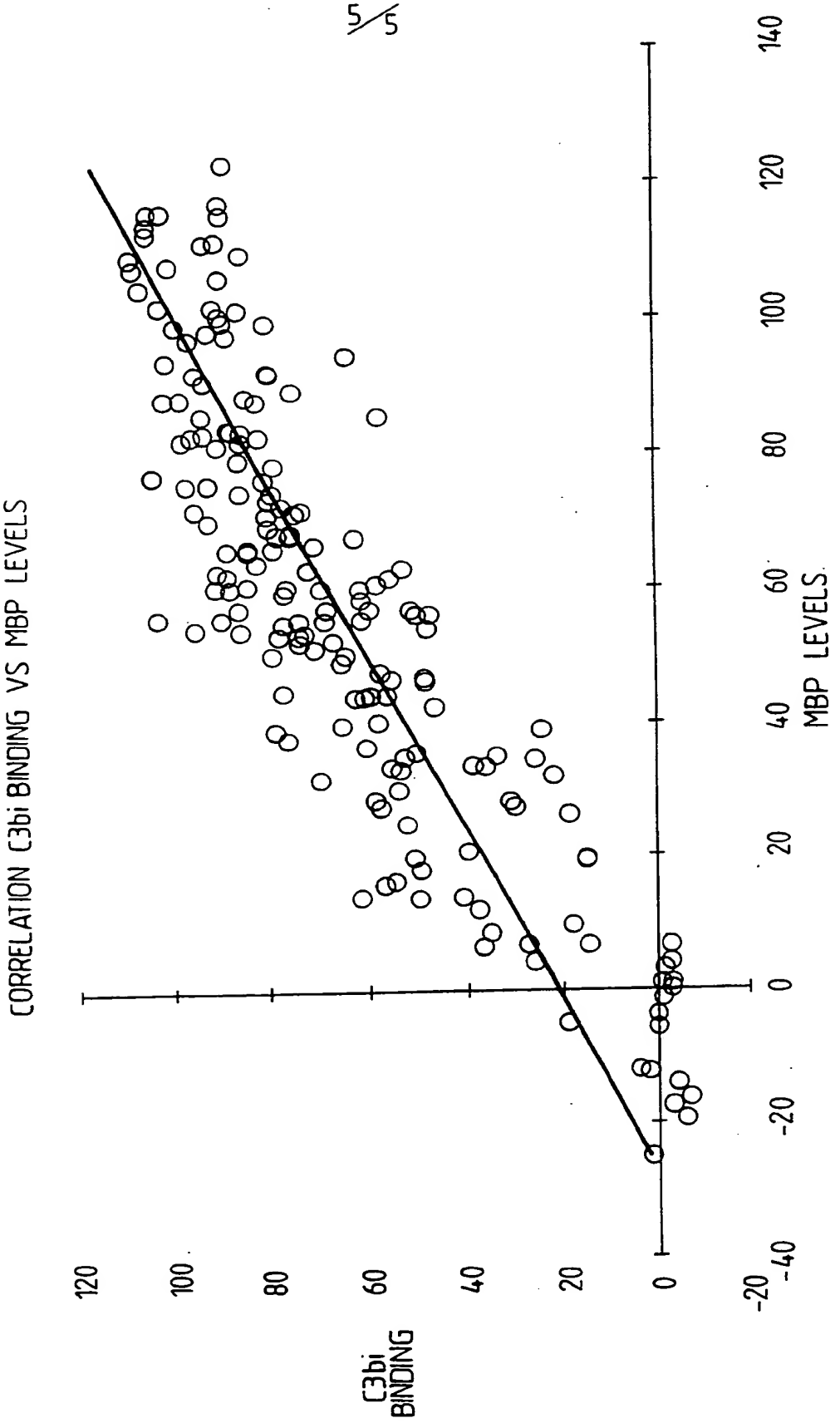


Fig.4c.



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/01564

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: G 01 N 33/68, G 01 N 33/545

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	WO, A, 89/01519 (CHILDREN'S HOSPITAL CORP.) 23 February 1989 see page 4, lines 12-22; page 14, lines 24-31; page 15, line 23 - page 16, line 2	1,2,11
Y	--	3-7,9
X,P	The Lancet, 25 November 1989, M. Super et al.: "Association of low levels of mannan-binding protein with a common defect of opsonisation", pages 1236-1239 see the whole article	1-3,5-7,9,11
A	--	4,8

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20th December 1990	23 JAN 1991
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Mme N. KUIPER

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X,P	The Journal of Immunology, vol. 144, no. 6, 15 March 1990, The American Association of Immunologists, J. Lu et al.: "Binding of the pentamer/hexamer forms of mannan-binding protein to zymosan activates the proenzyme $C1r_2C1s_2$ complex, of the classical pathway of complement, without involvement of C1q", pages 2287-2294 see the abstract; page 2288, column 2; page 2289, column 2; page 2291, column 2 - page 2293, column 1	1,2,4-6,8,9, 11
A	--	3,7
Y,P	Clinical and Experimental Immunology, vol. 79, no. 2, February 1990, Blackwell Scientific Publications, M. Super et al.: "The level of mannan-binding protein regulates the binding of complement-derived opsonins to mannan and zymosan at low serum concentrations", pages 144-150 see the whole article	3-7,9
A	-- The Journal of Experimental Medicine, vol. 169, no. 5, 1 May 1989, M. Kuhlman et al.: "The human mannose-binding protein functions as an opsonin", pages 1733-1745 see pages 1733-1735 cited in the application	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9001564 SA 41242

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 15/01/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8901519	23-02-89	AU-A- 2426288	09-03-89
		EP-A- 0375736	04-07-90